Exhibit 2: De Vries et al., *Eur. J. Biochem.* 224:613-622 (1994)

Eur. J. Biochem. 224, 613–622 (1994)

The single-stranded-DNA-binding proteins (SSB) of *Proteus mirabilis* and *Serratia marcescens*

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(Received March 25/June 2, 1994) – EJB 94 0414/2

The single-stranded-DNA-binding (SSB) proteins from *Proteus mirabilis* and *Serratia marcescens* were purified from overproducing *Escherichia coli* strains, which were devoid of their own *ssb* gene. The strains harboured an *endA* insertion mutation and a *xonA* mutation resulting in the absence of endonuclease I and exonuclease I activities from the preparations. The amino acid sequences of the SSB of all three species are nearly identical in the N-terminal parts of the proteins that contain the DNA-binding domain, but differ in the C-terminal parts. Both proteins have an apparent binding-site size of 65 and 35 nucleotides at high and low salt concentrations, respectively. The association-rate constant for binding to poly(dT) is $3.2\times10^8\,M^{-1}\,s^{-1}$ for *P. mirabilis* SSB (*Pmi*SSB) and $3.4\times10^6\,M^{-1}\,s^{-1}$ for *S. marcescens* SSB (*Sma*SSB). These binding parameters are very similar to those of *E. coli* SSB (EcoSSB). The structural similarity of the proteins is also documented by the finding that they can exchange subunits among each other to form mixed tetramers.

The transcriptional regulation of the *ssb* and *uvrA* genes from *P. mirabilis* and *S. marcescens* in SOS-induced *E. coli* cells was studied using *lacZ* fusions. While the *uvrA* genes were inducible, there was no induction of the *ssb* genes transcribed divergently from the *uvrA* genes. Apparently, regions with nucleotide sequence similarity to the *E. coli* SOS-box preceding the *ssb* genes of *P. mirabilis* and *S. marcescens* had no gross effect on the transcription. Studies on growth of the cells and recovery from ultraviolet damage indicate that the heterologous SSB proteins support DNA replication and recombinational DNA repair of *E. coli* with the same efficiency as the *E. coli* SSB protein. Interactions with other *E. coli* proteins involved in these processes either do not occur, or are not impeded.

Single-stranded-DNA-binding (SSB) proteins are important components of the macromolecular DNA metabolism of all kinds of organisms from viruses to vertebrates. Most of our knowledge about these proteins comes from studies on two non-related representatives, the gene 32 protein of phage T4 (T4 gp32) and the *Escherichia coli* SSB protein (*Eco*SSB). *Eco*SSB is essential and has been shown to be involved in DNA replication, DNA repair and genetic recombination (for a review, see Meyer and Laine, 1990). By binding of SSB, single-stranded DNA is prevented from forming secondary structures and becomes resistant to nucleases.

Binding of SSB to single-stranded nucleic acids can easily be monitored by the intrinsic tryptophan fluorescence of the protein. For *Eco*SSB at 0.3 M NaCl it was found that 65 nucleotides of poly(dT) are required to saturate a single tetramer and lead to a fluorescence quench of 90% (Lohman and Overman, 1985). Lowering the NaCl concentration leads to a change in binding modes, and at 0.05 M NaCl an apparent binding-site size of 33 nucleotides is observed with a fluorescence quench of 50% (Lohman and Overman, 1985). At 0.3 M NaCl the protein binds to the single-stranded poly(dT) in an almost-diffusion-controlled reaction (Urbanke and Schaper, 1990). For a review of biophysical properties of *Eco*SSB see Lohman et al. (1988), Greipel et al. (1989), and Lohman and Bujalowski (1990).

SSB proteins with a partial amino acid sequence identity to *Eco*SSB have been found to be encoded by a number of transmissible plasmids like the *E. coli* F-factor (Chase et al., 1983). Some of them were shown to complement *ssb* mutants of *E. coli* (Golub and Low, 1985, 1986; Howland et al., 1989; Porter and Black, 1991). The biological role of the plasmid SSB is still unknown.

We have recently reported the cloning and sequencing of two further bacterial *ssb* genes in addition to the *E. coli* gene, namely those of *Serratia marcescens* and *Proteus mirabilis* (de Vries and Wackernagel, 1993, 1994). In evolutionary history, these species have separated from *E. coli* about 200 million and 400 million years ago (Ochman and Wilson, 1987), and represent a relatively close and a most distant

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Abbreviations. SSB, single-stranded-DNA-binding protein; *Eco*SSB, SSB protein from *Escherichia coli*; *Pmi*SSB, SSB protein from *Proteus mirabilis*; *Sma*SSB, SSB protein from *Serratia marcescens*; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-glactosidase.

relative of *E. coli* among the enterobacteria. The SSB proteins of these species have 89% (*S. marcescens*) and 81% (*P. mirabilis*) amino acid sequence identity with the *E. coli* SSB.

Several authors have studied a possible regulation of the *ssb* gene of *E. coli* by the LexA repressor. Transcription from the $ssbP_1$ promoter, which is adjacent to the SOS box of the *uvrA* gene (located close to *ssb* and transcribed in the opposite direction as *ssb*), was inducible, but was accompanied by a decreased transcription from the two non-inducible promoters, $ssbP_{N1}$ and $ssbP_{N2}$ (Brandsma et al., 1985). Correspondingly, the relative rate of synthesis of SSB increased slowly, but there was no increase in the level of SSB measured in cell extracts (Perrino et al., 1987). In *P. mirabilis* and *S. marcescens*, the *ssb-uvrA* region is organized similarly to that in *E. coli*. In the *ssb* promoter regions of both organisms a region with nucleotide sequence similarity to the *E. coli* SOS consensus sequence was previously identified, which is not present in the corresponding region in *E. coli* (de Vries and Wackernagel, 1993; 1994).

In this study we investigated the regulation of transcription of the *ssb* and *uvrA* genes of *P. mirabilis* and *S. marcescens* cloned in *E. coli*, particularly a possible effect of the SOS-box-like sequences was examined. Some physiological properties of an *E. coli ssb*-deletion mutant containing the SSB proteins from *P. mirabilis* (*Pmi*SSB) or *S. marcescens* (*Sma*SSB) were also studied. Reactions of the purified proteins with single-stranded nucleic acids were investigated using physicochemical methods. Further, the compatibility of SSB protomers from the different organisms was examined in a subunit-exchange test. In addition, the amino acid sequences of the enterobacterial proteins are compared to those of plasmid-encoded SSB. The data are discussed with regard to the biological roles of SSB proteins in DNA metabolism.

MATERIALS AND METHODS

Purification of SSB proteins

*Pmi*SSB and *Sma*SSB were purified from the newly constructed strain BT270 which carries a *xonA2* mutation (Phillips et al., 1988) and an *endA* null mutation (Table 1; Jekel and Wackernagel, 1994). In this strain the tetracycline-resistance determinant from pBR322 was inserted into the *NsiI* site of the *endA* gene. The strain is therefore deficient for exonuclease I and endonuclease I and carries the chromosomal *ssb* deletion of RDP268 (Porter et al., 1990). The strain requires an ssb^+ helper plasmid for growth. The helper plasmid pACYC*ssb* (Porter et al., 1990) was replaced by pSBH2e or pSBH4e (Table 1) which carry the *ssb* genes of *P. mirabilis* and *S. marcescens*, respectively. This was achieved by plasmid transformation and subsequent isolation of chloramphenicol-sensitive segregants, which had lost pACYC*ssb*. The replacement was confirmed by restriction analysis of plasmid DNA isolated from the cells. *Eco*SSB was isolated from JM103 F^- harbouring pSBH5e (Table 1).

The SSB proteins were purified by the method of Lohman et al. (1986a) with minor modifications from cells grown in a 10-l fermenter (Braun). The preparations were about 99% pure as determined by SDS/PAGE and staining with Silver Stain (Bio-Rad). The *Pmi*SSB and *Sma*SSB isolated from strain BT270 were free of nuclease activity on linear single-stranded and double-stranded M13mp19 DNA up to a concentration of 2 μg/μl (the highest concentration tested), as determined by agarose-gel electrophoresis of DNA, that was incubated with different amounts of the proteins in 25 mM Tris, pH 7.5, 12 mM $MgCl_2$, 3.5% glycerol for 2 h at 37°C. The proteins were stable in storage buffer [20 mM potassium phosphate, pH 7.5, 1 M NaCl, 1 mM EDTA, 60% (by vol.) glycerol] at −20°C for at least 6 months.

Construction of *lacZ* fusions for determination of the promoter strength

Several restriction fragments of the *ssb-uvrA* intergenic regions from *E. coli*, *S. marcescens* and *P. mirabilis* (Fig. 1) were cloned in both orientations into the *Sma*I site of the promoter test plasmid pTL61T (Linn and St Pierre, 1990). Where necessary, single-stranded overhangs were removed by mung-bean nuclease digestion (Gibco BRL). In one orientation, the transcription towards *ssb* is measured (pTL-sb plasmid series), in the other the transcription towards *uvrA* (pTL-uv plasmid series). The fragments with the complete promoter-operator regions of the three species were also cloned into the single-copy-number vector λTL61 (Linn and St Pierre, 1990) using the *Eco*RI- and *Xba*I sites of the identical multiple-cloning sites in both vectors. In the phage vector, *Eco*RI and *Xba*I are unique sites. Since the single-stranded ends produced by these enzymes are not complementary, the inserts were ligated into the arms in the same orientation as they had in the plasmid vector (with respect to the *lacZ* gene). The correct location and orientation of DNA fragments in the clones was verified by restriction analysis.

For the purpose of measuring the SOS inducibility of the cloned promoters in the λ vector, the phage *21* cI repressor of this vector had to be replaced by a repressor which is not cleaved upon SOS induction by mitomycin C. This was achieved by crossing the λcI857 gene (Sussman and Jacob, 1962) coding for an SOS-induction-resistant, thermosensitive cI repressor into the promoter-containing phages. *E. coli* C600 was used as host for the phage crosses. The descendants of the cross were plated on 5-bromo-4-chloro-3-indolyl-β-D-glactosidase (X-gal)-plates with soft-agar (0.7%) containing 120 μg X-gal/ml and *E. coli* BT124 (Table 1) as indicator. After incubation for 16 h at 37°C, clear blue plaques were isolated and purified twice as single plaques. The correct size and orientation of the inserts was verified by restriction of the phage DNA with *Eco*RI and *Xba*I. Corresponding to the plasmids, the promoter test phages were termed λTL-sb20, λTL-sb40 and λTL-uv20, λTL-uv40, λTL-uv50 (Fig. 1).

Lysogen formation

Formation of lysogens of BT124 and BT148 (Table 1) was carried out as described (Linn and St Pierre, 1990). Lysogens were identified on agar plates containing X-gal (40 μg/ml). To distinguish monolysogens from multiple lysogens, a terminase excision test (Mousset and Thomas, 1969) was carried out: exponentially growing cultures (2 ml; 29°C) of the lysogens at a titre of 2×10^8/ml were brought to 10 mM $MgSO_4$ and superinfected with the heteroimmune λTL61 (phage *21* immunity) at an infection multiplicity of two. The cultures were further aerated for 2 h. Then two drops of chloroform were added and incubation was continued for 10 min. Descendant phages with λ immunity were titred on λTL61 lysogens of *E. coli* C600 (Table 1). Monolysogens produced about 5×10^6 phage, while multiple lysogens produced about 5×10^7 phage with λcI857 immunity/ml in this test. The β-galactosidase activity of identified monolysogens (about

Table 1. Strains, phage and plasmids used in this study.

E. coli strains, phage and plasmids	Relevant genotype/description	Source or reference
RDP268	Δ(*ssb-uvrA*); only viable with an *ssb*⁻ helper plasmid	Porter et al. (1990)
BT124	Δ*lac*(U169); as GW1000, but *recA*⁺	Kenyon and Walker (1980)
BT148	Δ*lac*(U169); as GW1000, but *recA*⁺, *lexA3*	Kenyon and Walker (1980)
JM103 (F⁻)	*endA1*; the strain was cured of the F factor by electroporation	Yanish-Perron et al. (1985)
BT270	*ssb*::Km^R, *xonA2*, *endA*::Tc^R; only viable with an *ssb*⁺ helper plasmid	this work
λTL61	promoter cloning phage vector	Linn and St Pierre (1990)
λcI857	cI857-repressor (thermosensitive)	Sussman and Jacob (1962)
λTL61-cI857	same as λTL61, but with the λcI857-repressor	this work
pTL61T	pBR322-derived promoter cloning plasmid vector	Linn and St Pierre (1990)
pRE432	mini-F-derived single-copy-number cosmid vector	de Vries and Wackernagel (1992)
pBluescript II KS+	high-copy-number vector	Stratagene, Heidelberg, Germany
pACYC*ssb*	*E. coli ssb*⁺ gene in pACYC184	Porter et al. (1990)
pSBH2e	*P. mirabilis ssb*⁺ gene in pBluescript II KS+	de Vries and Wackernagel (1994)
pSBH4e	*S. marcescens ssb*⁺ gene in pBluescript KS+	de Vries and Wackernagel (1993)
pSBH5e	*E. coli ssb*⁺ gene in pBluescript II KS+	de Vries and Wackernagel (1993)
pSBL4	*S. marcescens ssb*⁺ gene in pRE432	de Vries and Wackernagel (1993)
pSBL5	*E. coli ssb*⁺ gene in pRE432	de Vries and Wackernagel (1993)
pTL-sb20	*P. mirabilis*, bp 338–599 (*RsaI*–*RsaI*)	this work
pTL-sb21	*P. mirabilis*, bp 480–623 (*MseI*–*MseI*)	this work
pTL-sb22	*P. mirabilis*, bp 338–494 (*RsaI*–*Sau*3AI)	this work
pTL-uv20	*P. mirabilis*, bp 599–338 (*RsaI*–*RsaI*)	this work
pTL-uv22	*P. mirabilis*, bp 494–338 (*Sau*3AI-*RsaI*)	this work
pTL-sb40	*S. marcescens*, bp 290–550 (*Sau*3AI-*Sau*3AI)	this work
pTL-sb42	*S. marcescens*, bp 131–437 (*Hae*III–*RsaI*)	this work
pTL-uv40	*S. marcescens*, bp 550–290 (*Sau*3AI–*Sau*3AI)	this work
pTL-uv42	*S. marcescens*, bp 437–131 (*RsaI*–*Hae*III)	this work
pTL-sb50	*E. coli*, bp 85 in *uvrA* sequence (Husain et al., 1986) to bp 276 in *ssb* sequence (Sancar et al., 1981) (*NarI*–*PvuII*, total length 524 bp)	this work
pTL-uv50	*E. coli*, bp 276 in *ssb* sequence (Sancar et al., 1981) to bp 85 in *uvrA* sequence (Husain et al., 1986) (*PvuII*–*NarI*; total length 524 bp)	this work

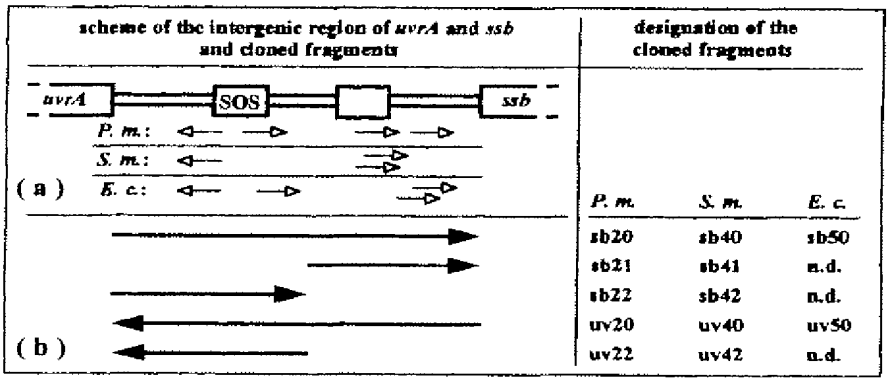

Fig. 1. Scheme of the intergenic region between *uvrA* and *ssb* in *P. mirabilis*, *S. marcescens* and *E. coli*. (a) The start sites of the open reading frames of *uvrA* and *ssb* are indicated. SOS represents the SOS boxes regulating the transcription of the *uvrA* genes. A second region with lower similarity to the *E. coli* SOS box consensus sequence is indicated by an empty box (in *E. coli*, only the left of these two regions is present). The location and direction of transcription of promoters in the three species is indicated by the open arrows. (b) The filled arrows indicate the fragments cloned into pTL61T and the direction of transcription measured (i. e. the orientation of the cloned fragment in pTL61T). The designation of the cloned fragments is given for *P. mirabilis* (*P. m.*), *S. marcescens* (*S. m.*) or *E. coli* (*E. c.*); n.d., not determined.

370 U/A_{600} of 1 at a titre of 10^8/ml) and multiple lysogens of BT124 for λTL-sb40 (about 700 U/A_{600} of 1) confirmed the reliability of the test. As expected, the lysogens were non-viable at 42°C due to induction of the prophage by inactivation of the cI857 repressor. The *Eco*RI-restriction pattern of the DNA of phage released by thermal induction was identical to that of the DNA of the phage used for lysogen formation.

Determination of ultraviolet sensitivity and generation time

The ultraviolet sensitivity and generation time of exponentially growing cultures of *E. coli* were determined as described (de Vries and Wackernagel, 1993, 1994).

Determination of β-galactosidase activity and SOS induction

The β-galactosidase activity of all transcriptional fusions was determined by the method of Kenyon et al. (1982). The SOS response was induced by the addition of mitomycin C (Sigma) to the exponentially growing cultures (final concentration of 0.5 μg/ml in *lexA*$^+$ strains; 0.1 μg/ml in *lexA3* strains).

Binding of SSB proteins to single-stranded nucleic acids

Fluorescence measurements, stopped-flow experiments, and melting curves were carried out in a standard buffer containing 0.02 M potassium phosphate, pH 7.4, 0.1 mM EDTA and NaCl as indicated. In fluorescence experiments 100 ppm Tween20 were added.

Fluorescence measurements were carried out in a Schoeffel RRS1000 spectrofluorimeter at 22°C. Excitation was at 295 nm and emission was observed at 350 nm.

In inverse fluorescence titrations a mixture of protein and nucleic acid was added to the protein such that at all points the protein concentration was kept constant. Inverse titrations are often evaluated with respect to an apparent binding-site size using the intersection of the limiting slopes at low and high single-stranded DNA/protein ratios. In all titrations the absorbance of the solution was below 0.05 at the excitation wavelength to avoid inner filter effects. After each addition the solution was allowed to equilibrate for 60–600 s until no fluorescence change with time could be observed. Theoretical binding isotherms were calculated using the model of Schwarz and Watanabe (1983) for the binding of a multidentate ligand to a linear polymer as described earlier (Curth et al., 1993).

Melting curves were measured in a DMR10 (Zeiss) spectrophotometer in standard buffer and 0.1 M NaCl as described earlier (Augustyns et al., 1991). Temperature slopes were 20 K/h and no difference could be observed between heating and cooling curves.

Stopped-flow experiments were performed at 22°C in a modified version of a Durrum-Gibson stopped-flow apparatus and were evaluated using a model for irreversible binding of a multidentate ligand to a linear polymer, as described previously (Urbanke and Schaper, 1990).

Poly(dT) and poly(dA-dT) were purchased from Pharmacia. Poly(dT) had an average length of 1400 bases/strand as calculated from the sedimentation and diffusion coefficients measured by analytical ultracentrifugation and dynamic light scattering, respectively. Poly(dA-dT) showed a broad distribution in length ranging from several hundred to 10000 bp as judged from agarose-gel electrophoresis. Concentrations of poly(dT) and poly(dA-dT) were determined photometrically with a molar absorption coefficient of 8600 $M^{-1}cm^{-1}$ at maximum (Urbanke and Schaper, 1990) and 6700 $M^{-1}cm^{-1}$ at 260 nm (Williams et al., 1983), respectively, and are given in monomer units throughout the text.

Protein concentrations are given in units of tetramers throughout the text and were determined using a molar absorption coefficient at 280 nm of 113000 $M^{-1}cm^{-1}$ as determined previously (Lohman and Overman, 1985) for *Eco*SSB. Since the predicted amino acid sequences indicate that *Eco*SSB, *Pmi*SSB and *Sma*SSB have no differences in aromatic amino acid composition no differences in the molar absorption coefficients at 280 nm are to be expected.

Amino acid sequencing

Amino acid sequencing was performed with the purified proteins with an Applied Biosystems 477A protein sequencer.

Subunit-exchange measurements

The proteins were diluted from stock solutions to a concentration of 100 μM with SBP buffer [20 mM potassium phosphate, pH 7.5, 0.5 M NaCl, 1 mM EDTA, 20% (by vol.) glycerol]. Equal volumes of these solutions were mixed and incubated at room temperature for the indicated times. Samples were diluted with equal volumes of loading buffer [50% (by vol.) glycerol, 0.1% bromphenol blue]. Samples containing 125 pmol of each protein were loaded onto an 0.5% agarose gel and electrophoresed in 20 mM Tris, pH 7.8, 0.4 mM NaAc, 0.2 mM EDTA as described earlier (Lohman et al., 1986b). The gel was stained with Coomassie Blue for 1 min and destained in 7% (by vol.) acetic acid at room temperature for several days.

RESULTS AND DISCUSSION

Analysis of the expression and regulation of *ssb* and *uvrA* genes

The analysis of the nucleotide sequence of the intergenic region between *ssb* and *uvrA* had revealed two sequences in both *S. marcescens* and *P. mirabilis*, with similarity to the *E. coli* SOS box (de Vries and Wackernagel, 1993, 1994; EMBL accession numbers X65079, X65080). SOS boxes are operators where the LexA repressor binds regulating the expression of genes of the SOS regulon (Walker, 1984). In both species the nucleotide sequence similarity of the SOS-box-like sequence that overlaps with the *uvrA* promoter to the *E. coli* SOS consensus sequence (TACTGTATATA-ATACAGTA) was high [TACTGGATATCCATTCAGGT (*S. marcescens*), TACTGTATATCCATTCAGCT (*P. mirabilis*)], and less high for the other one that overlaps with the *ssb* promoter region [GTGTGGTTTCGTACTCAGCG (*S. marcescens*), TCCTGTTCTCAATGGAGAA (*P. mirabilis*)].

To determine the strength of the promoters and to identify a possible regulation by the *LexA* repressor, transcriptional *lacZ* fusions were constructed and expressed in *E. coli*. Fragments containing the entire intergenic region as well as smaller fragments containing either of the two SOS-box-like sequences were subcloned into the promoter-analysis vector pTL61T (Linn and St Pierre, 1990). Fig. 1 shows a scheme

Table 2. β-galactosidase activity of transcriptional fusions of *ssb* and *uvrA* promoters in pTL61T. The designation of the plasmids is described in Fig. 1. The determination of β-galactosidase activity was performed by the method of Kenyon et al. (1982). The induction factors were calculated by division of induced by uninduced β-galactosidase activity of the *lexA*$^+$ strain, separately for 1 h and 2 h after induction. The values in parentheses were determined similarly for the uninducible *lexA3* mutant. The data are means from three experiments (± the standard deviation).

Plasmid	β-galactosidase activity of uninduced cultures after induction for			Induction factor in *lexA*$^+$ (in *lexA*3) after induction for	
	0 h	1 h	2 h	1h	2 h
	1000 U/A_{600} of 1				
pTL-sb20	9.0 ± 0.9	8.9 ± 0.8	12.0 ± 0.8	0.8 ± 0.1 (0.7)	0.6 ± 0.2 (0.7)
pTL-sb21	9.2 ± 2.2	9.9 ± 0.2	14.6 ± 0.5	0.9 ± 0.1 (0.8)	1.2 ± 0.1 (0.5)
pTL-sb22	6.0 ± 1.6	5.6 ± 0.7	9.4 ± 2.1	1.6 ± 0.1 (1.2)	1.4 ± 0.2 (0.7)
pTL-uv20	4.8 ± 0.1	4.2 ± 0.7	5.5 ± 1.1	1.3 ± 0.1 (1.0)	1.5 ± 0.1 (0.6)
pTL-uv22	5.0 ± 0.5	4.8 ± 0.2	8.1 ± 0.3	1.4 ± 0.3 (0.4)	1.1 ± 0.1 (0.6)
pTL-sb40	7.6 ± 0.8	9.3 ± 0.1	14.5 ± 1.0	1.0 ± 0.1 (0.8)	1.1 ± 0.1 (0.7)
pTL-sb41	9.0 ± 0.1	10.7 ± 0.4	15.3 ± 0.2	0.9 ± 0.1 (1.0)	1.0 ± 0.0 (0.6)
pTL-sb42	0.4 ± 0.0	0.6 ± 0.0	0.9 ± 0.0	1.1 ± 0.1 (0.7)	1.0 ± 0.0 (0.7)
pTL-uv40	6.6 ± 0.1	7.0 ± 0.6	9.6 ± 1.1	2.2 ± 0.4 (0.6)	2.6 ± 0.3 (0.6)
pTL-uv42	8.3 ± 1.5	6.8 ± 0.4	11.1 ± 0.7	2.0 ± 0.5 (0.6)	2.1 ± 0.0 (0.7)
pTL-sb50	7.1 ± 0.6	9.2 ± 0.4	15.4 ± 0.9	1.2 ± 0.2 (1.1)	0.9 ± 0.3 (0.9)
pTL-uv50	1.3 ± 0.2	1.3 ± 0.4	4.6 ± 0.4	3.2 ± 0.2 (1.2)	2.2 ± 0.3 (1.1)
pTL61T	0.7	0.7	1.2	1.0 (1.0)	0.9 (0.9)

of the various cloned regions and the designation of the plasmids containing the fragments. With each of these plasmids the β-galactosidase activity was determined under non-induced conditions and after SOS induction by mitomycin C (see Materials and Methods), both in a *lexA*$^+$ strain and a non-inducible *lexA*3 mutant.

The β-galactosidase activity obtained with the pTL-sb plasmids from *P. mirabilis*, *S. marcescens* and *E. coli* in uninduced cells at a titre of about 10^8/ml was in the range of about 6–9000 U/A_{600} of 1 with all of the pTL-sb plasmids (Table 2). Only pTL-sb42 gave a low level of activity as the promoterless vector pTL61T. It was concluded that no transcription towards *ssb* was initiated from the *S. marcescens* region containing the *uvrA* promoter and SOS operator sequences. In this respect, *S. marcescens* differs from *E. coli*, where transcription towards *ssb* is initiated by a promoter adjacent to the *uvrA* SOS box (Brandsma et al., 1985).

Transcription towards *uvrA* produced 1300 U/A_{600} of 1 from the *E. coli* promoter (pTL-uv50), 4800 from the *P. mirabilis* promoter (pTL-uv20) and 6600 from the *S. marcescens* promoter in pTL-uv40. Similar results were obtained when the smaller fragments with only the *uvrA* promoters were employed (Table 2). 2 h after induction with mitomycin C the cultures entered the stationary phase, and the β-galactosidase activity increased gradually with all of the plasmids.

The induction factors (level of induced activity divided by uninduced level) obtained with mitomycin C induction are shown in the right two columns of Table 2. In a *lexA*$^-$ background the *P. mirabilis ssb* promoter in pTL-sb22 was inducible (by a factor of 1.6). The *lacZ* expression from the larger fragment contained in pTL-sb20 was not inducible, rather it was slightly reduced after application of mitomycin C. A similar observation was made in *E. coli*, where the induction of the $ssbP_1$ promoter was accompanied by a decrease of the transcription from $ssbP_{N1}$ and $ssbP_{N2}$ (Brandsma et al., 1985). The expression towards *ssb* in the other constructs with promoters of *E. coli*, *P. mirabilis* and *S. marcescens* was not inducible (Table 2).

In contrast, transcription towards *uvrA* was induced by mitomycin C in constructs with the *uvrA* promoters of *P. mirabilis* (by a factor of 1.5), *S. marcescens* (by a factor of 2.6) and *E. coli* (by a factor of 3.2), which is in accord with published data on *E. coli* (Kenyon and Walker, 1980). The inducibility was abolished in *lexA*3 cells (in which the LexA repressor is not cleaved due to a mutation), indicating that the SOS boxes at the *uvrA* promoters of *P. mirabilis* and *S. marcescens* were recognized by the *E. coli* LexA repressor.

The observed absent or low SOS induction of the *ssb* genes may result from the use of the multicopy vector pTL61T, which might titrate the LexA repressor by the high number of SOS boxes/cell. Therefore, the insert fragments with the complete promoter regions were also cloned into the single-copy-number vector λTL61. However, the results of β-galactosidase-activity determinations with the phage vector (Table 3) after SOS induction gave an induction pattern in *lexA*$^+$ and *lexA3* cells similar to that obtained with the multicopy vector. Again, the *ssb*-promoter regions of *S. marcescens* and *P. mirabilis* (λTL-sb20 and λTL-sb40) did not display any inducibility.

The results suggest that the SOS-box-like sequences preceding the *ssb* genes of *P. mirabilis* and *S. marcescens* have no gross effect on the *ssb* gene expression in SOS-induced *E. coli* cells. Possibly, the similarity of the SOS-box-like sequences of *P. mirabilis* and *S. marcescens* to the *E. coli* SOS consensus sequence is not sufficiently high to allow efficient binding of the *E. coli* LexA repressor. This would leave open the possibility that these putative SOS boxes have a regulatory function in *P. mirabilis* and *S. marcescens*. Expression of the *nucA* gene of *S. marcescens*, which is also preceded by an SOS-box-like sequence, is inducible in *S. marcescens*, but not in *E. coli* (Ball et al., 1990). The *uvrA* genes of *P. mirabilis* and *S. marcescens* are inducible in *E. coli*, although not to the same degree as the *E. coli uvrA* gene (Tables 2 and 3). The lower induction factors could result from deviations in the nucleotide sequence of the SOS boxes in *S. marcescens* and *P. mirabilis* from the *E. coli* SOS box consensus

Table 3. β-galactosidase activity of transcriptional fusions of *ssb* and *uvrA* promoters in the single-copy-number vector λTL61T. For further details, see the legend to Table 2.

Lysogen	β-galactosidase-activity of uninduced cultures after induction for				Induction factor in *lexA*⁺ (in *lexA*3) after induction for		
	0 h	1 h	2 h	3 h	1 h	2 h	3 h
	U/A_{600} of 1						
λTL-sb20	400 ± 34	380 ± 36	470 ± 36	560 ± 88	0.9 ± 0.1 (0.9)	0.9 ± 0.0 (1.0)	0.9 ± 0.1 (1.0)
λTL-uv20	76 ± 04	86 ± 03	110 ± 07	140 ± 07	1.3 ± 0.1 (1.0)	1.4 ± 0.1 (1.0)	1.5 ± 0.0 (1.0)
λTL-sb40	370 ± 45	310 ± 32	380 ± 17	450 ± 40	1.1 ± 0.1 (1.1)	1.0 ± 0.1 (0.9)	0.8 ± 0.2 (0.9)
λTL-uv40	300 ± 09	280 ± 04	320 ± 04	330 ± 10	1.8 ± 0.0 (1.0)	3.1 ± 0.0 (1.0)	3.6 ± 0.1 (1.0)
λTL-uv50	140 ± 01	110 ± 10	130 ± 15	140 ± 12	1.7 ± 0.1 (0.8)	3.2 ± 0.1 (1.0)	4.9 ± 0.1 (1.0)
λTL61-cI857	71	58	78	84	1.1 (1.0)	1.0 (1.0)	1.0 (1.0)

sequence. The SOS regulons of the three enterobacterial species are apparently similar, but they are probably not identical. The determination of the nucleotide sequences of more SOS boxes from *S. marcescens* and *P. mirabilis* would be necessary for establishing the SOS consensus sequences for these species.

Comparison of the amino acid sequences of chromosomally and plasmid-encoded SSB proteins

In *E. coli* the N-terminal methionine residue of *Eco*SSB is cleaved post-translationally (Sancar et al., 1981). From the determination of the N-terminal amino acid sequences of *Pmi*SSB and *Sma*SSB expressed in *E. coli* the same cleavage of methionine can be shown. The alignment of the amino acid sequences of the bacterial SSB proteins with those of five SSB proteins encoded by transmissible plasmids (Fig. 2) indicates that all eight proteins are nearly identical up to position 88. Amino acids that are presumed to be involved in tetramer formation (His55; Williams et al., 1984; Curth et al., 1991) or DNA binding (Trp40, Trp54, Phe60, Trp88; Merril et al., 1984; Casas-Finet et al., 1987; Khamis et al., 1987; Curth et al., 1993) of the *Eco*SSB are found at identical positions within all eight proteins. The C-terminal third is more variable within each group, and between both groups the amino acid sequence similarity is low. From position 114–177, there are only three stretches of 4, 3 and 5 amino acids, starting at positions 136, 149, and 173, that are highly conserved in all eight SSB proteins. Other regions are highly conserved within a group, but are completely different between both groups. This pattern of regions with high and low amino acid sequence similarity may reflect the evolutionary relatedness of SSB, i. e. differences would indicate positions not essential for the structure and functioning of the proteins. However, it is possible that the regions with low or no sequence similarity between bacterial and plasmid-encoded SSB have different functions in both groups (Chase et al., 1983).

Compared to the other SSB proteins, the *E. coli* SSB contains an apparent insertion of six amino acids at positions 126–131 (the *S. marcescens* protein has a smaller insertion of three amino acids at the same position). This insertion could be a duplication of the nucleotide sequence that immediately follows, encoding the amino acid sequence GWGQPQ. Only three nucleotides are different, which results in the replacement of W by G. The duplication produces a further copy of the sequence GQPQ, which is one of the three conserved regions in the C-terminus among all eight proteins.

DNA binding by the SSB proteins from *S. marcescens* and *P. mirabilis*

One of the most important biophysical properties of SSB proteins is their ability to destabilize double-stranded nucleic acids. Fig. 3 shows the destabilization of alternating poly(dA-dT) by both *Sma*SSB and *Pmi*SSB. From the relative transition midpoints one sees directly that both proteins drastically destabilize poly(dA-dT) and that the double-strand-destabilizing ability of *Sma*SSB is somewhat higher than that of *Pmi*SSB. Under the same conditions the transition midpoint of *Eco*SSB is at 48 °C which is almost equal to that of *Pmi*SSB.

For *Eco*SSB the binding to single-stranded nucleic acids has been investigated with various substrates with the affinity decreasing in the order poly(dT), phage single-stranded DNA (fd, ΦX174), poly(dC), poly(dC,dT), poly(dA,dC), poly(rU), poly(dA), polyribonucleic acids (Overman et al., 1988; Greipel et. al, 1989). Since poly(dT) shows the strongest binding to *Eco*SSB it is especially suited to measure the binding-site size. However, this strong binding prevents the determination of binding affinities. In inverse titrations at 0.3 M NaCl in standard buffer 65 nucleotides of poly(dT) are covered by a single *Eco*SSB tetramer leading to a fluorescence decrease of the protein by 90% (Lohman and Overman, 1985; Bujalowski and Lohman, 1986; Bujalowski et al., 1988). Fig. 4 shows fluorescence titrations for *Sma*SSB and *Pmi*SSB under the same conditions. Binding-site size and fluorescence quench are similar to *Eco*SSB with only minor differences. For *Eco*SSB a binding-mode transition has been shown when changing the ionic environment from high salt (≥0.3 M NaCl) to low salt (≤0.05 M NaCl) where the apparent binding-site size decreases from 65 to 33 nucleotides/tetramer (Lohman and Overman, 1985; Bujalowski et al., 1988). A similar transition is observed for *Pmi*SSB and *Sma*SSB (Fig. 5).

The kinetics of binding to poly(dT) can be observed by fluorescence changes in a stopped-flow experiment. Using a kinetic model for the binding of a multidentate ligand to a linear polymer described earlier (Urbanke and Schaper, 1990) the association-rate constants for the binding of a protein tetramer to its binding site was determined to be $(3.2 \pm 0.4) \times 10^5\ M^{-1}\ s^{-1}$ for *Pmi*SSB and $(3.4 \pm 0.4) \times 10^6\ M^{-1}\ s^{-1}$ for *Sma*SSB. These values do not differ significantly

```
                1         10         20         30         40         50         60         70
E. coli         ASRGVNKVILVGNLGQDPEVRYMPNGGAVANITLATSESWRDKATGEMKEQTEWHRVVLFGKLAEVASEY
S. m.           ASRGVNKVILVGNLGQDPEVRYMPHGGAVANITLATSESWRDKATGEQKEKTEWHRVVLFGKLAEVAGEY
P. m.           ASRGVNKVILIGNLGQDPEIRYMPSGGAVANLTLATSESWRDKQTGEMKEKTEWHRVVIFGKLAEIAGEY
                * ** ******* ** ****** ********   ********* **** * ********************
F               AVRGINKVILVGRLGKDPEVRYIPNGGAVANLQVATSESWRDKQTGEMREQTEWHRVVLFGKLAEVAGEC
pIP231a         AVRGINKVILVGRLGKDPEVRYIPNGGAVANLQVATSETWRDKQTGKMREQTEWHRVVLFGKLAEVAGEY
pIP71a          AVRGINKVILVGRLGKDPEVRYIPNGGAVANLQVATSESWRDKQTGEIREQTEWHRVVLFGKLAEVAGEY
R64             SARGINKVILVGRLGNDPEVRYIPNGGAVANLQVATSESWRDKQTGEMREQTEWHRVVLFGKLAEVAGEY
ColIb-P9        SARGINKVILVGRLGNDPEVRYIPNGGAVANLQVATSESWRDKQTGEMREQTEWHRVVLFGKLAEVAGEY

                          80         90         100        110        120        130        140
E. coli         LRKGSQVYIEGQLRTRKWTDQSGQDRYTTEVVVNVGGTMQMLGGRQGGGAPAGGNIGGGQPQGGWGQPQQ
S. m.           LRKGSQVYIEGSLQTRKWQDQSGQDRYTTEIVVNVGGTMQMLGGRQGGGAPAGQSAGG...QGGWGQPQQ
P. m.           LRKGSQVYIEGQLQTRKWQDQSGQDRYSTEVVVNIGGTMQMLGGRGGQDNAPSQG......QGGWGQPQQ
                **** ******** ** *    *  ** **  *   *******   *                *   ****
F               LRKGAQVYIEGQLRTRSW.KDNGITRYVTEILVKTTGTMQMLVRAAGAQTQPEEG......QQFSGQPQP
pIP231a         LRKGAQVYIEGQLRTRSW.KDNGITRYVTEILVKTTGTMQMLGRAAGAQTQPEEG......QQFSAQPQP
pIP71a          LRKGAQVYIEGQLRTRSW.KDNGITRYVTEILVKTTGTMQMLGRAAGTQTQPEEA......QQFSGQPQP
R64             LRKGAQVYIEGQLRTRSW.DDNGITRYITEILVKTTGTMQMLGSAPQQNAQAQPK......PQQNGQPQS
ColIb-P9        LRKGAQVYIEGQLRTRSW.DDNGITRYITEILVKTTGTMQMLGSAPQQNAQAQPK......PQQNGQPQS

                150        160        170   177     # aa      aa identity
E. coli         PQGGNQ..FSGGAQ....SRFQQ..SAPAAPSNEPPMDFDDDIPF     177        100 %
S. m.           PQGGNQ..FSGGQQ...QSRFAQ..NSAPATSNEPPMDFDDDIPF     175         89 %
P. m.           PQASQQ..FSGGAP....SRFAQQPAAAPAPSNEPPMDFDDDIPF     173         81 %
                       ***      * *      * *      * *****
F               EPQAEAGTKKGGAKTKGRGRKAAQPEPQPQPPEGDDYGFSDDIPF     178         60 %
pIP231a         EPQSEAGTKKGGAKTKGRGRKVAQPEPQLQPPEGDDYGFSDDIPF     178         60 %
pIP71a          ESQPEP..KKGGAKTKGRERKAAQPEPR.QPSEPAY.DFDDDIPF     174         62 %
R64             ADAT....KKGGAKTKGRERKAAQPEPQPQTPEGEDYGFSDDIPF     174         59 %
ColIb-P9        ADAT....KKGGAKTKGRGRKAAQPEPQPQTPEGEDYGFSDDIPF     174         61 %
```

Fig. 2. Alignment of SSB proteins from enterobacteria and conjugative plasmids F (Chase et al., 1983), pIP231a, pIP71a, R64 (Ruvolo et al., 1991) and ColIb-P9 (Howland et al., 1989). The alignment was performed with the program MULTALIN (Corpet, 1988) using the Dayhoff-matrix (Dayhoff, 1978) and a gap-penalty of 8. The sequences are arranged in two groups (bacterial and plasmid SSB). In each group, conserved amino acids are shown in bold type. (*), Positions of identity between both groups (amino acids which are identical among of at least 50% of the members of each group). The number of amino acids of each protein (# aa) and the percentage of amino acids identical to the *E. coli* protein (aa identity) are given.

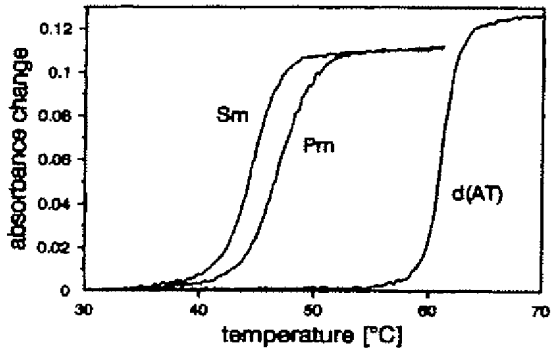

Fig. 3. Melting profiles of poly(dA-dT) and its complexes with *Pmi*SSB and *Sma*SSB. 38.0 μM poly(dA-dT) alone [d(AT)] and with 1.3 μM *Pmi*SSB (Pm) or *Sma*SSB (Sm) respectively in standard buffer at 0.1 M NaCl. Transition midpoints are at 61°C [d(AT)], 47°C (Pm), and 45°C (Sm). Absorbance changes were measured at 260 nm.

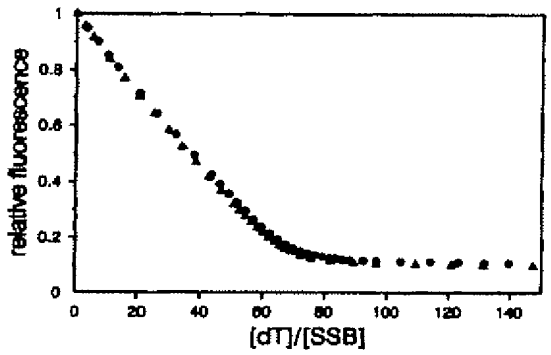

Fig. 4. Inverse fluorescence titration of *Pmi*SSB (●) and *Sma*SSB (▲) with poly(dT). 0.31 μM SSB protein (tetramer) were titrated with poly(dT) at 0.3 M NaCl in standard buffer. The binding-site size and fluorescence quench were calculated to be 66 nucleotides/tetramer and 89% for *Pmi*SSB, and 65 nucleotides/tetramer and 90% for *Sma*SSB.

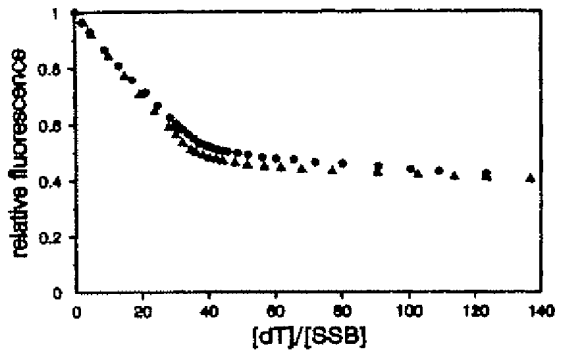

Fig. 5. Inverse fluorescence titration of 0.18 μM *Pmi*SSB (●) and 0.15 μM *Sma*SSB (▲) with poly(dT) at 1 mM NaCl, 1 mM potassium phosphate, pH 7.4, 0.1 mM EDTA and 100 ppm Tween20. The binding-site size was estimated from the limiting slopes to be 37 residues for *Pmi*SSB and 36 residues for *Sma*SSB, respectively.

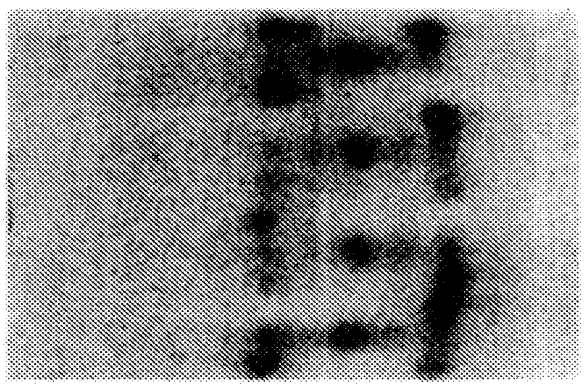

Fig. 6. Subunit exchanges between different SSB tetramers. Agarose-gel electrophoresis of different proteins mixed together in equimolar amounts and incubated at 23°C for 65 h, or put immediately onto the gel (0 h), and unmixed proteins. From top to bottom: *Pmi*SSB with *Eco*SSB, 0 h; *Pmi*SSB with *Eco*SSB, 65 h; *Eco*SSB alone; *Pmi*SSB alone; *Pmi*SSB with *Sma*SSB, 65 h; *Pmi*SSB with *Sma*SSB, 0 h; *Sma*SSB alone; *Sma*SSB with *Eco*SSB(Δ116−167), 65 h; *Sma*SSB with *Eco*SSB(Δ116−167), 0 h; *Eco*SSB(Δ116−167) alone; *Eco*SSB with *Eco*SSB(Δ116−167), 65 h; *Eco*SSB with *Eco*SSB(Δ116−167), 0 h.

from the values determined for *Eco*SSB (Urbanke and Schaper, 1990).

Subunit exchange

Evidence for the structural and functional similarity of the various SSB proteins would be their ability to form mixed tetramers with each other. Different electrophoretic mobilities in a non-denaturing agarose gel can be used to separate homogeneous and mixed tetramers. SSB proteins, due to the highly charged C-terminus, have a net negative charge. *Pmi*SSB having one acidic residue more than *Eco*SSB and *Sma*SSB can be separated from the latter two proteins. To obtain differences in electrophoretic mobility between *Eco*SSB and *Sma*SSB we used a deletion mutant of *Eco*SSB (*Eco*SSBΔ116−167) having the same net charge as wild-type *Eco*SSB but a reduced molecular mass and thus higher electrophoretic mobility. *Eco*SSBΔ116−167 cannot be separated, however, from *Pmi*SSB.

As shown in Fig. 6 the different SSB proteins form mixed tetramers when incubated for 65 h at room temperature. In a time-resolved exchange experiment between *Pmi*SSB and *Sma*SSB the first mixed tetramers appeared after 12 h (data not shown). There is no preference for the homotetramers in the exchange experiments. We therefore conclude that the mechanism of interaction in forming the tetramer must be virtually identical in all SSB proteins investigated. If the exchange was statistically random, a 1 : 1 mixture of SSB proteins A and B should form five types of tetramers (A_4, A_3B, A_2B_2, AB_3 and B_4) in a binominal distribution 1 : 4 : 6 : 4 : 1. This behaviour has been shown for lactate dehydrogenase (Markert, 1963), which is a tetramer formed by two electrophoretically distinguishable subunits. The relative intensities of the different bands in Fig. 6 show, however, that the amount of the A_2B_2 type is far more than 50% larger than that of the corresponding A_3B or AB_3 heterotetramers. This observation can be explained if the exchange is preferentially between dimers and not monomers. This explanation agrees well with the fact that *Eco*SSB crystals show a D_2 symmetry (Hilgenfeld et al., 1984) and suggest a dimer-of-dimers structure for the tetramer. Tetramers which contain two dimers of two different species could then very well be more prominent than 1 : 3 mixed tetramers. One might speculate that different structural domains in the monomer might be responsible for dimer and tetramer formation. Experimental results which show no detectable amounts of dimers in the dissociation of *Eco*SSBH55Y (*ssb-1*) (Bujalowski and Lohman, 1991) do not contradict this interpretation since in both cases the dimeric state is only a transition state in either dissociation or subunit exchange and is not significantly populated compared to monomers or tetramers. Furthermore the compatibility of the SSB subunits from different species differing mainly in their C-terminal sequence and that of *Eco*SSBΔ116−167 indicates that tetramer formation is independent of the C-terminal amino acids.

Complementation of *E. coli* mutants by the *ssb* genes of *P. mirabilis* and *S. marcescens*

The construction of an *ssb* deletion mutant, which is viable only when bearing a helper plasmid with an *ssb* gene (Porter et al., 1990) has made complementation studies with heterologous *ssb* genes possible (no *Eco*SSB is present). Previously, the presence of *ssb-1* or *ssb-113* alleles (point mutations) in *E. coli* precluded correct interspecies complementation studies. The deletion mutant was used to determine the generation time and ultraviolet resistance of *E. coli* cells with *Pmi*SSB or *Sma*SSB, as measures for the function of these proteins in DNA replication and repair.

The generation time of *E. coli* RDP268 (Δ*ssb*) carrying the *S. marcescens ssb* gene on the single-copy-number plasmid pSBL4 (Table 1) was 20.7±0.8 min, compared to 20.9±0.6 min with the *E. coli ssb* gene on the same vector (plasmid pSBL5; Table 1). With *Pmi*SSB the generation time was 20.5±0.5 min (de Vries and Wackernagel, 1994). Thus, both heterologous SSB proteins can efficiently replace the *Eco*SSB in its function in DNA replication.

The ultraviolet sensitivity of *E. coli* RDP268 with pSBL4 or pSBL5 is shown in Fig. 7. In the *ssb-uvrA* deletion mutant, the recovery from ultraviolet damage depends on recombinational repair due to the lack of the UvrA protein required for excision repair. *Sma*SSB promotes DNA repair in *uvrA* cells

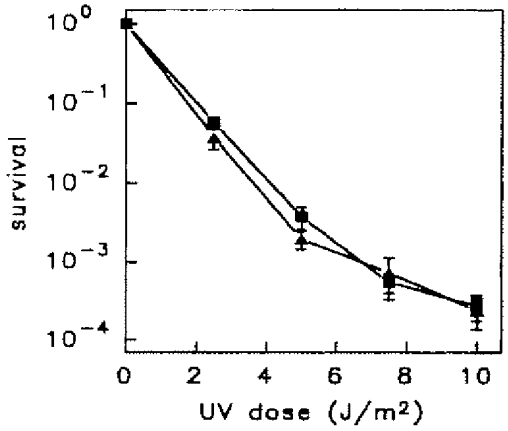

Fig. 7. Ultraviolet sensitivity of *E. coli uvrA-ssb* (RDP268; Table 1) with the *ssb* genes of *S. marcescens* (located on pSBL4; ▲) or *E. coli* (located on pSBL5; ■). The curves are means from three experiments. The error bars indicate standard deviations.

with the same efficiency as *Eco*SSB. This was also the case for *Pmi*SSB (de Vries and Wackernagel, 1994). Thus, the differences in the amino acid sequences of the three enterobacterial SSB have no measurable effect on replication and recombinational repair in *E. coli*. The high similarity of the physicochemical properties of the proteins (see above) is probably the basis for the full support of replication and recombinational DNA repair in *E. coli* by the three proteins.

If interactions of SSB with other components of the macromolecular DNA metabolism exist, they seem not to be species specific, because the function of the *Eco*SSB can be fully replaced by *Sma*SSB or *Pmi*SSB. In previous experiments, in which other enzymes of the macromolecular DNA metabolism were exchanged between the three species, evidence for species-specific interactions between RecA and RecBCD enzymes was obtained (Rinken et al., 1991; de Vries and Wackernagel, 1992). The tolerance for non-cognate SSB proteins as observed here would also be expected if no interactions of SSB with other enzymes exist.

The authors thank Dr R. D. Porter (Pennsylvania State University) and Dr T. Linn (University of Western Ontario) for bacterial strains and plasmids, B. Thoms (Universität Oldenburg) for the construction of *E. coli* strains, and Dr U. Curth (Medizinische Hochschule Hannover) for valuable discussions and help with the melting experiments. This work was supported by the *Deutsche Forschungsgemeinschaft* and the *Fonds der Chemischen Industrie*.

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